Reduction of Furin-Nicked *Pseudomonas* Exotoxin A: An Unfolding Story

Marian L. McKee and David J. FitzGerald*

Biotherapy Section, Laboratory of Molecular Biology, Division of Basic Science, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892-4255

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ABSTRACT: Upon entering mammalian cells, *Pseudomonas* exotoxin A (PE) is proteolytically processed by furin to produce an N-terminal fragment of 28 kDa and a C-terminal fragment of 37 kDa. Cleavage is followed by the reduction of a key disulfide bond (cysteines 265−287). This combination of proteolysis and reduction releases the 37 kDa C-terminal fragment, which then translocates to the cytosol where it ADP-ribosylates elongation factor 2 and inhibits protein synthesis. To investigate toxin reduction, furin-nicked PE or a hypercleavable mutant, PEW281A, was subjected to various treatments and then analyzed for fragment production. Reduction was evident only when unfolding conditions and a reducing agent were applied. Thermal unfolding of PE, as evidenced by changes in α-helical content and increased sensitivity to trypsin, rendered nicked toxin susceptible to protein disulfide isomerase- (PDI-) mediated reduction. When subcellular fractions from toxin-sensitive cells were incubated with nicked PE, toxin unfolding and reducing activities were present in the membrane fraction but not the soluble fraction. These data indicate that PE reduction is a two-step process: unfolding that allows access to the Cys265−287 disulfide bond, followed by reduction of the sulfur−sulfur bond by PDI or a PDI-like enzyme. With regard to cellular processing, we propose that the toxin's three-dimensional structure retains a "closed" conformation that restricts solvent access to the Cys265−287 disulfide bond until after a cell-mediated unfolding event.

Pseudomonas exotoxin A (PE)[1] is a three-domain ADP-ribosylating toxin secreted by *Pseudomonas aeruginosa*. The domain structure of PE is as follows: domain Ia (aa 1−252) mediates receptor binding; domain II (aa 253−365) harbors the protease processing site, delimited by the Cys265−287 disulfide bond, and has sequences that mediate translocation; domain Ib (aa 366−394) has no known function but may serve to enhance toxin stability; and finally, domain III (aa 395−613) has ADP-ribosylating activity and a KDEL-like sequence at the end of the protein (*1*−*3*). When the toxin interacts with mammalian cells, PE uses the low-density lipoprotein receptor-related protein (LRP) as its receptor for endocytic uptake (*4*−*6*). Once inside cells, PE is cleaved by furin in an acidic endocytic compartment to produce two peptide fragments that remain covalently linked by the Cys265−287 disulfide bond (*7*−*10*). Because only the C-terminal 37 kDa fragment reaches the cytosol, the Cys265−287 disulfide bond must be reduced prior to translocation. After reduction and translocation, this fragment ADP-ribosylates elongation factor 2, resulting in the cessation of protein synthesis and cell death (*11*−*13*). Currently, neither the cellular location nor the mechanism of reduction of this key disulfide bond is known.

PE is secreted from *Ps. aeruginosa* as a proenzyme. Structure−function analysis of various PE mutants together with information derived from the toxin's crystal structure has indicated the importance of both interdomain and intradomain interactions in maintaining this state. Among others, residues 246, 247, and 249 of domain Ia play an important role in maintaining the proenzyme state (*14*). Also, PE lacking the Cys265−287 disulfide bond is less active than its wild-type counterpart and less stable (*15*). When chemical denaturants are used, it requires 8 M urea and a reducing agent to unfold native PE and reveal its enzymatic activity (*16*). In viewing the PE crystal structure it is apparent that the Cys265−287 is not exposed to solvent (*1*). It is therefore of some interest to determine how the toxin is processed within cells to allow access to this bond.

Previous biophysical studies of native PE structure indicated that incubation of PE with acidic buffers increases the exposure of hydrophobic residues (*17*, *18*). Jiang and London (*19*) have proposed that PE goes through two distinct pH-induced conformational changes. The first state is a conformational change that does not reveal hydrophobic residues, whereas the second state reveals hydrophobic sequences that may interact with phospholipids (*19*−*21*). Also, furin cleavage is absolutely dependent on the continued presence of an acidic environment. Exposure to low pH followed by return to neutral pH did not allow cleavage at the protease recognition site (*9*). Additionally, Corboy and Draper (*22*) found that pH-elevating drugs protected cells from both nicked PE and native PE. To what extent low pH can and does mediate the changes needed to allow intracellular processing is currently not clear.

* Corresponding author: 37 Convent Dr., MSC 4255, Bethesda, MD 20892-4255. Phone (301) 496-9457; Fax (301) 402-1969; E-mail djpf@helix.nih.gov.

[1] Abbreviations: PE, *Pseudomonas* exotoxin A; DTT, dithiothreitol; LRP, low-density lipoprotein receptor-related protein; HSP, high-speed pellet; PDI, protein disulfide isomerase, EC 5.3.4.1; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GSH, reduced glutathione; GSSG, glutathione disulfide; NEM, *N*-ethylmaleimide; rPE37, recombinant *Pseudomonas* exotoxin A 37-kDa fragment; ER, endoplasmic reticulum.

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The presence of an endoplasmic reticulum (ER) retention-like sequence at the C-terminus of PE is absolutely required for toxicity (*14*). This suggests that PE traffics either directly or indirectly from the endosome to the ER and in so doing may use a retrograde version of the secretion pathway for its journey to the cytosol (the retrograde transport of toxins to the ER is reviewed in ref *23*). The involvement of the ER allows one to speculate that the cellular components normally used to fold proteins and oxidize disulfide bonds could be used in reverse to unfold the toxin and reduce the disulfide bond, and in so doing release PE's enzymatically active fragment for translocation. Because protein disulfide isomerase (PDI, EC 5.3.4.1) is a multifunctional enzyme (reviewed in refs *24* and *25*), is found abundantly in the ER, and has been shown to participate in the reduction of other toxins, we have examined its role in PE reduction.

In this study we report on conditions that can reduce furin-cleaved PE. For reduction to occur, toxin structure must first be altered, presumably to allow cellular reductants and enzymes access to the disulfide bond. While we have not yet identified a cell-associated unfolding component, evidence is presented that PDI can function in the reductive process and is a likely component in cell-mediated reduction of PE.

EXPERIMENTAL PROCEDURES

Reagents. Oxidized and reduced glutathione, *N*-ethylmaleimide, dithiothreitol, and ATP were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture media and reagents were from BioWhittaker and Gibco−BRL. Protein concentration determinations were made with the Pierce Coomassie Plus protein assay reagent system (Pierce, Rockford, IL). Sequencing-grade trypsin was purchased from Roche Molecular Biochemicals.

Cells. Chinese hamster ovary (CHO) and murine fibroblast L929 cells were obtained from ATCC (Rockville, MD). Cells were propagated in DMEM (L929) or RPMI-1640 (CHO) supplemented with 5% fetal bovine serum, glutamine (1 mM), and penicillin (50 units/mL) and streptomycin (50 μg/mL).

Toxin Expression and Purification. PE, PEW281A, and rPE37 toxins were produced in *Escherichia coli* BL21/λDE3 from plasmids pVC45D, pAla3, or pDF1, respectively, under control of an inducible T7 promoter system as described (*26*). PE, PEW281A, and rPE37 were isolated from the bacterial periplasm and purified by ion-exchange and gel-filtration chromatography (*27*).

Preparation of Crude Membrane Fractions. Subcellular fractions of cultured mammalian cells were obtained as described in ref *28* with modifications (*29*, *30*). Cells were grown to near confluence in Nunc bioassay dishes (24 × 24 × 1.8 cm). The monolayer was washed once with ice-cold phosphate-buffered saline, pH 7.4 (PBS), with 5% BSA, followed by three washes with cold PBS. Cells were harvested by scraping into a conical centrifuge tube and centrifuging at 200*g* in a Beckman RT6000. Cells were resuspended in homogenization buffer (HB; 250 mM sucrose and 3 mM imidazole, pH 7.4) with 0.5 mM EDTA and 1 mM AEBSF (PefaBloc, Boerhinger) and they were disrupted with 20 strokes of a tight-fitting Dounce tissue homogenizer (Wheaton, Millville, NJ). To remove unbroken cells and nuclei (low-speed pellet, LSP), the sample was centrifuged at 800*g* at 4 °C in a Sorvall RT6000C tabletop centrifuge. This low-speed supernatant (LSS or postnuclear supernatant, PNS) was centrifuged again at 100000*g* for 90 min at 4 °C in a 50.2 Ti rotor in a Beckman LS5 ultracentrifuge to obtain the crude membrane fraction (high-speed pellet, HSP) and the cytosolic fraction (high-speed supernatant, HSS). The HSP fraction was resuspended in PBS with 1 mM PefaBloc. The protein concentration of each fraction was determined. Fractions were stored concentrated at −80 °C until diluted in PBS for the reduction assay.

In Vitro Assay of Toxin Unfolding. Furin-nicked PE (5 μg) was incubated in the presence of 100 mM HEPES, pH 7.5, 0.1 mM EDTA, and 50 mM KCl at the temperatures indicated (Figure 4) for 30 min. At the end of the incubation, trypsin was added in the same buffer to each reaction and the incubation was continued at 37 °C for all samples. The reactions were stopped by addition of sample buffer containing β-mercaptoethanol and subjected to reducing SDS−PAGE. The resulting proteins were visualized by Coomassie blue staining with Gelcode blue stain reagent (Pierce).

For analysis of HSP-mediated unfolding, 100 ng of nicked toxin was mixed with 5 μg (total protein) of HSP in the presence 100 mM HEPES, pH 7.5, 0.1 mM EDTA, and 50 mM KCl of for 30 min at 37 °C. The reactions were treated with trypsin as described above. The molar ratio of trypsin for the HSP assay was calculated on the basis of the total protein in the assay (HSP plus toxin). After separation of the resultant products by reducing SDS−PAGE, the proteins were transferred to nitrocellulose for immunodetection with affinity-purified polyclonal rabbit anti-PE serum. The immunoreactive species were detected by chemiluminescence on Hyperfilm (ECL, Amersham)

In Vitro Assay of Toxin Reduction. PE was treated with purified recombinant furin in 0.2 M sodium acetate, pH 5.5, with 1 mM calcium chloride overnight to obtain furin-nicked toxin for all reduction assays. For chemical reduction experiments, 3 μg of nicked toxin was incubated with 1−100 mM DTT in 50 mM Tris, pH 7.5. Following a 30 min incubation at room temperature, nonreducing SDS−PAGE sample buffer was directly added to the sample or the treated toxin was precipitated with 6% trichloroacetic acid on ice and washed with ice-cold acetone before addition of sample buffer. Reduction products were separated immediately on nonreducing SDS−PAGE without prior boiling and protein bands were visualized by staining with Coomassie Brilliant Blue.

For analysis of HSP-mediated reduction, 50 ng of nicked toxin was mixed with cellular fractions in the presence of 0.2 M sodium acetate (pH 5.5), 1 mM calcium chloride, 1 mM reduced glutathione (GSH), 0.2 mM oxidized glutathione (GSSG), and 50 mM ATP for 90 min at 37 °C unless otherwise specified. The reactions were quenched with 10 mM *N*-ethylmaleimide (NEM) and then mixed with nonreducing SDS−PAGE sample buffer. After separation of the products on nonreducing SDS−PAGE, proteins were transferred to nitrocellulose for immunodetection with the monoclonal antibody M40-1 or polyclonal rabbit anti-PE serum. The immunoreactive species were detected by chemiluminescence (ECL, Amersham) on Hyperfilm (Amersham).

In Vitro PDI Assay. In vitro reduction of PE by purified bovine liver PDI (PanVera Corporation, Madison, WI) was assessed by incubating 50 ng of nicked toxin in the presence

of 5 μM PDI, 50 mM sodium acetate (pH 5.5) or 50 mM Tris (pH 7.5), 1 mM GSH, and 0.2 mM GSSG at room temperature. The reaction was quenched with 10 mM NEM and resolved by nonreducing SDS−PAGE as described. Visualization of reduction products was by immunodetection and chemiluminescence as described above.

Circular Dichroism Studies. Circular dichroism spectra were collected on a Jasco J-720 Spectropolarimeter with a 1-mm path length cell. Far-UV CD spectra (200−260 nm) were obtained in 0.1 nm increments with a 0.5 nm bandwidth and a 2 s time constant (50 nm/min average). The proteins were analyzed as a 2 μM sample in 10 mM monobasic potassium phosphate (pH 7.4), 250 μM EDTA, and 150 mM potassium chloride. Temperature scans were performed by scanning continuously from 5 to 80 °C at a scan rate of 1 °C min^{-1}. Each spectrum was digitally smoothed by use of the Savitsky−Golay algorithm, corrected for concentration, and normalized to units of mean residue weight ellipticity (Θ_{MRW}) by use of the following relationship:

$$\Theta_{MRW} = \Theta_{obs}[M_{r(monomer)}/n_{monomer}]/10dc \qquad (1)$$

Θ_{obs} is the observed ellipticity, $M_{r(monomer)}$ is the molecular weight of the monomer, $n_{monomer}$ is the number of amino acids in the monomer, d is the path length of the cell (centimeters), and c is the concentration of the sample in the cell (milligrams per milliliter).

RESULTS

Chemical Reduction of PE. The endoprotease furin cleaves PE in an arginine-rich loop between Arg279 and Gly280. After cleavage, the resulting fragments remained joined covalently by the disulfide bond between Cys265 and Cys287 (*8*, *9*). Reduction of this disulfide bond produces an N-terminal fragment of 28 kDa and a C-terminal fragment of 37 kDa. The 37 kDa fragment of PE translocates to the cytosol where it ADP-ribosylates elongation factor 2 (*7*). Leppla et al. (*16*) demonstrated that PE as a proenzyme must be treated with both a denaturant (i.e., urea or SDS) and a reductant to exhibit enzymatic activity. In this study, we sought to characterize the cellular components responsible for generating an enzymatically active fragment from intact PE.

To determine conditions suitable for PE reduction, the toxin was first cleaved with furin (*9*) and the nicked toxin was then subjected to increasing concentrations of various reducing agents (i.e., 1−100 mM DTT). At the end of the 30 min incubation, reaction mixtures were prepared for polyacrylamide gel electrophoretic analysis in one of two ways: one set of samples was subjected to TCA precipitation and the precipitate was washed with acetone to remove the low molecular weight reductants. Products were then analyzed by nonreducing SDS−PAGE (samples were not heated). The other set was analyzed by adding nonreducing SDS sample buffer directly to each reaction tube. In the samples where TCA was added and the reductant was removed, no fragments were generated when DTT was present up to 100 mM (Figure 1, TCA). However, in the samples with direct mixing of SDS and reductant, fragment production was detected at 1 mM DTT and above (Figure 1, no TCA). Because furin cleaves wild-type PE inefficiently, three bands are seen: unnicked PE and fragments corresponding to reduced PE28 and PE37 (Figure 1). Similar

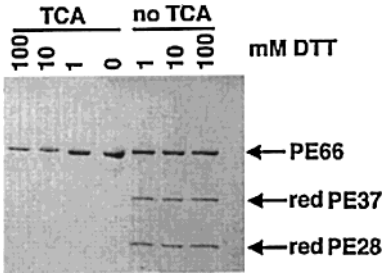

FIGURE 1: DTT-mediated reduction of PE. Furin-nicked PE was treated with 1, 10, or 100 mM DTT. Either DTT-treated toxin was precipitated with TCA to remove the reducing agent and then mixed with nonreducing SDS sample buffer (TCA), or nicked PE was treated with DTT and mixed directly with nonreducing SDS sample buffer (no TCA). Samples were analyzed by SDS−PAGE in the absence of reducing agent, stained with Coomassie brilliant blue G-250, and photographed. PE66, full-length PE; red PE37, C-terminal 37 kDa fragment; red PE28, N-terminal 28 kDa fragment.

results were obtained when either glutathione (GSH) or β-mercaptoethanol was used as the reducing agent (data not shown). By comparison, when nicked diphtheria toxin (DT) was treated with reducing agents and then precipitated with TCA, the intrachain disulfide bond was susceptible to direct chemical reduction at 10 mM DTT (data not shown).

Enzymatic Reduction of PE. In seeking to understand the cellular processes responsible for uncovering the Cys265−287 disulfide bond, we examined the role of known reduction systems under physiological conditions. Orlandi (*31*) reported that PDI was sufficient to mediate the reduction of membrane-bound, nicked cholera toxin A chain in mammalian cells. PDI, a major ER resident protein, has disulfide oxidizing activity (*24*, *25*) and may also have chaperone activities (*32*, *33*).

PDI completely isomerizes the disulfide bonds in ribonuclease A at 5 μM PDI in the presence of 50 mM Tris-HCl, 1 mM GSH, and 0.2 mM GSSG (final ratio 5:1). (*34*). Under these conditions, either furin-nicked PE (Figure 2A) or furin-nicked PEW281A (Figure 2B) was incubated at various temperatures. The hypercleavable mutant PEW281A (*35*) was used in these analyses, because native PE is only partially cleaved by furin. The inclusion of this mutant allowed us to better visualize the appearance of PE37 after reduction. PE is cleaved at the acidic pH found in endosomes, while PDI resides primarily in the neutral pH environment of the ER. Therefore, furin-nicked PE was mixed with PDI at either pH 5.5 (Figure 2A) or pH 7.0 (data not shown). Regardless of pH, PDI could not reduce nicked PE at either 22 or 37 °C (Figure 2A, +PDI, and data not shown). However, when nicked PE was incubated with PDI at 54 °C, reduction of PE was evident (Figure 2A, +PDI, 54 °C). Products were analyzed by nonreducing SDS−PAGE (without heating of the sample) followed by immunoblotting. Transferred proteins were probed with the monoclonal antibody M40-1, which recognizes a segment of PE between amino acids 290 and 308 (*36*) and reacts with full-sized PE (PE66) and the reduced 37 kDa fragment (PE37). PDI is a thermostable enzyme and retains much of its activity at temperatures up to and including 54 °C (*37*). In the absence of PDI but in the presence of glutathione, no PE37 was observed at temperatures below 65 °C (Figure 2A, −PDI). Some degradation of nicked PE occurred at 65 °C as evidenced by the decrease in total protein at 65 °C in the

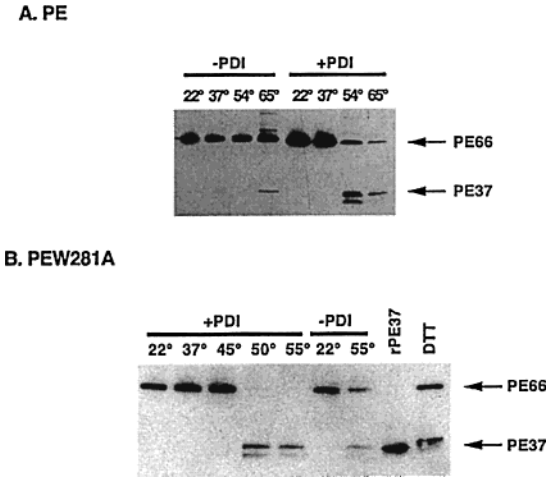

FIGURE 2: Protein unfolding is required for PDI-mediated reduction of PE and PEW281A. Nicked PE (A) or PEW281A (B) was incubated with (+PDI) or without (−PDI) 5 μM PDI in the presence of glutathione in sodium acetate buffer, pH 5.5, at increasing temperatures. After incubation, the reactions were terminated with 10 mM NEM, separated by nonreducing SDS−PAGE, transferred to nitrocellulose, reacted with M40-1, and detected by ECL. PE66, full-length PE; PE37, C-terminal fragment after reduction of the Cys265−287 disulfide bond; DTT, nicked toxin treated with 1 mM DTT in the presence of SDS.

presence of PDI (Figure 2A, 65 °C). When nicked PEW281A was incubated with PDI and glutathione at pH 5.5 (data not shown) or pH 7.0 (Figure 2B), reduced fragments were produced at 50 and 55 °C but not at ≤45 °C (Figure 2B). Unlike PE, heating PEW281A to 55 °C resulted in formation of a small amount of PE37 in the absence of PDI. For comparison, the migration of recombinant PE37 (rPE37) and fully nicked PE37, produced by the addition of DTT and SDS sample buffer, is shown (Figure 2B).

From these data we conclude that under physiological conditions PDI alone is not sufficient to mediate the reduction of nicked PE. The result suggests that an additional component is required in this process. When the 3-D structure of PE is viewed, it is apparent that the disulfide bond is not exposed to solvent (*1*). In addition, residues in domain Ia (amino acids 1−252) form hydrogen bonds with residues in domains Ib (amino acids 365−399) and domain III (amino acids 405−613) that stabilize the toxin in a "closed" conformation (*1*, *14*). Together with the studies of Leppla and others, these data point to the need for an unfolding step to allow reduction.

Thermal Unfolding of PE. We sought to determine how heating furin-nicked PE to 50 °C allowed for PDI-mediated reduction. In the crystal structure of PE the Cys265−287 disulfide bond joins the A helix to the B helix of domain II. Speculating that the disruption of one of these α-helical structures would be necessary for reductants to gain access to the disulfide bond, we performed thermal melt analysis using CD spectrophotometry. Because native PE is only partially cleaved by furin and because in all reduction assays PE and PEW281A behaved the same, we used the hypercleavable mutant PEW281A for these analyses (*35*). Far-UV spectra were obtained for unnicked PEW281A (Figure 3A) and furin-nicked PEW281A (Figure 3B) at 5 and 10 °C increments at neutral pH. Results indicated that there was a change in helix structure in both nicked and unnicked PE between 45 and 50 °C as monitored by changes at 222 nm.

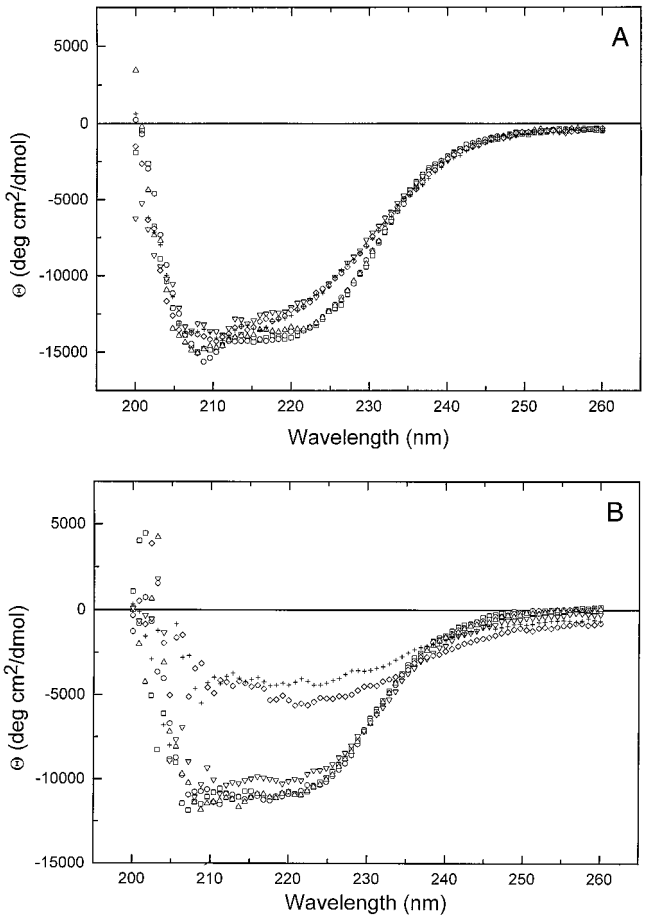

FIGURE 3: Nicked PEW281A undergoes a conformational change at 50 °C. Far-UV spectra (mean of 10 scans) of PEW281A unnicked (A) or nicked by furin (B) were obtained in potassium buffer, pH 7.4. Temperatures: 25 °C, □; 35 °C, ○; 45 °C, △; 50 °C, ▽; 55 °C, ◇; 65 °C, +.

A more dramatic structural change occurred between 50 and 55 °C after the toxin was nicked with furin (Figure 3B). These observations were further substantiated by the difference in the calculated T_m at 222 nm between unnicked and nicked PEW281A [T_m(unnicked) = 52.5 °C vs T_m(nicked) = 50.4 °C] (data not shown). The observed structural alterations due to elevated temperature were irreversible (data not shown). The change in the helical structure of PE at elevated temperature was consistent with the observed requirement for thermal unfolding of PE before PDI could access and reduce the Cys265−287 disulfide bond (Figure 2). We propose that PDI gained access to the buried disulfide bond when the helical structure of nicked toxin was altered due to heating to 50 °C.

Unfolded PE Is Susceptible to Trypsin. While CD spectroscopy is useful for gathering structural information about highly purified proteins, it is less useful when protein mixtures are being analyzed. In the latter situation, protein unfolding can be detected by increased susceptibility to protease degradation. Therefore, we analyzed PE unfolding by monitoring the products of trypsin treatment. To look for evidence of unfolding, either furin-nicked PE or PEW281A was heated to 22, 37, or 54 °C and then mixed at 37 °C with trypsin. Final trypsin-to-toxin ratio was either 1:2 or 1:10, and products were analyzed by reducing SDS−PAGE (Figure 4). Results indicated that only treatment at 54 °C produced an unfolded state. As mentioned above, the treat-

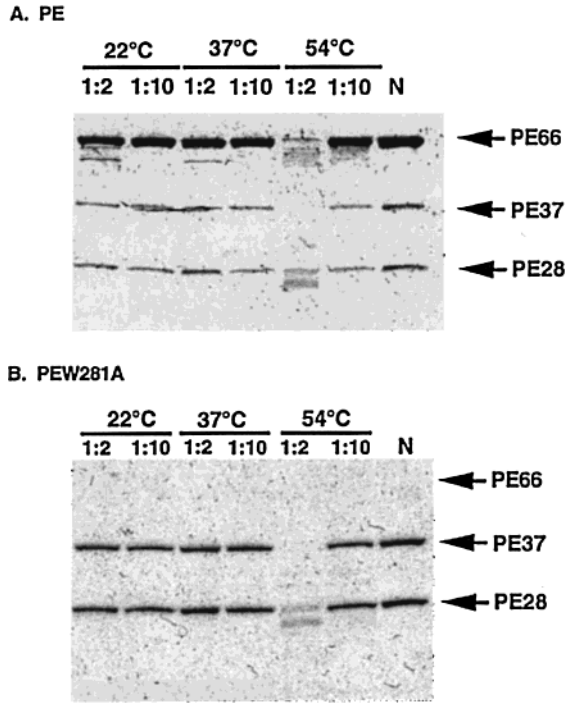

FIGURE 4: Unfolded nicked PE and PEW281A are trypsin-sensitive. Furin-nicked PE (A) or PEW281A (B) was incubated at the indicated temperatures in 100 mM HEPES, pH 7.5, 0.1 mM EDTA, and 50 mM KCl before digestion with trypsin (1:2 or 1:10 molar ratio of trypsin:toxin) at 37 °C. The samples were separated by reducing SDS−PAGE and visualized by Coomassie staining. N, furin-nicked toxin with no trypsin treatment.

ment of native PE with furin produces a mixture of nicked and unnicked protein. Interestingly, there was no evidence that PEW281A was more susceptible to trypsin degradation than the native toxin protein (compare panel A to panel B in Figure 4). At 22 and 37 °C and at the 1:2 ratio of trypsin to toxin, an additional band is seen at ~55 kDa, most likely the product of trypsin cleavage at Arg490 (*38*). However, at 54 °C in the presence of a 1:2 ratio of trypsin to toxin, there is almost complete loss of wild-type toxin and evidence of multiple degradation products (Figure 4A). At 54 °C in the presence of the higher concentration of trypsin, PEW281A was totally degraded (Figure 4B). At the 1:10 ratio, there was little evidence of increased susceptibility to protease for either toxin.

Toxin Unfolding and Reduction by Cellular Fractions. Because toxin processing occurs intracellularly, we next sought the cellular components and conditions that could unfold the toxin and allow reduction of the Cys265−287 disulfide bond. Intact mammalian cells were disrupted and then fractionated into membrane and soluble components by differential centrifugation. Equivalent amounts of protein from each fraction were incubated with nicked PE (Figure 5A). Reductive activity was assessed in the presence of 0.2 M sodium acetate (pH 5.5), 1 mM calcium, 0.2 mM oxidized glutathione, 1 mM reduced glutathione, and 50 mM ATP at 37 °C for 90 min. At the end of the incubation period, 10 mM *N*-ethylmaleimide (NEM) was added as a quenching agent prior to the addition of nonreducing SDS−PAGE sample buffer. Products were analyzed by nonreducing SDS−PAGE (without heating of the sample) followed by immunoblotting, and transferred proteins were probed with M40-1. Most of the reductive activity was found in the high-speed pellet (HSP). Activity from samples prepared from CHO and L929 cells is shown in Figure 5A. Some reductive activity was associated with the low-speed pellet (LSP), which contains intact and broken cells as well as the membrane fraction. None was associated with the high-speed supernatant (HSS), which represents the cell cytosol. Reductive activity was eliminated either by boiling the HSP or by pretreating it with 10 mM NEM (data not shown).

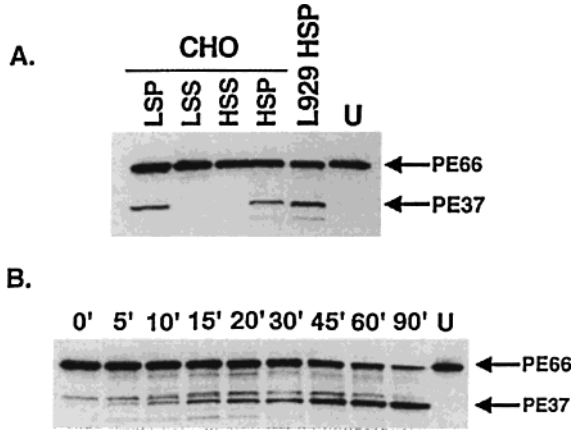

FIGURE 5: Toxin reducing activity localizes to the high-speed pellet (HSP). (A) Furin-cleaved PE was incubated with cellular fractions derived from either CHO or L929 cells. Samples were incubated at pH 5.5 in the presence of GSH/GSSG, calcium, and ATP for 1.5 h at 37 °C. To determine the extent of reduction, reactions were analyzed by nonreducing SDS−PAGE, transferred to nitrocellulose, and visualized by immunoblot analysis with the monoclonal antibody M40-1. LSP, low-speed pellet; LSS, low-speed supernatant; HSS, high-speed supernatant; HSP, high-speed pellet; PE66, full-length PE; PE37, C-terminal fragment after reduction. (B) Nicked PE was incubated with HSP from L929 cells as in panel A, but aliquots were removed at the times indicated, and the reduction reaction was stopped by the addition of 10 mM NEM. Samples were resolved by nonreducing SDS−PAGE and immunoblotted with M40-1 as described.

To address the progression of HSP-mediated PE reduction over time, samples were taken at time points from 0 to 90 min and evaluated by nonreducing SDS−PAGE followed by immunoblotting as described. Reduction of nicked PE proceeded through at least three intermediates over 90 min (Figure 5B). At 90 min, the reduction reaction had reached completion.

To determine whether the HSP fraction contained factors that could mediate toxin unfolding, 5 μg of HSP was incubated with 50 ng of nicked PE in the presence of HEPES buffer (pH 7.5) at 37 °C for 30 min before trypsin was added in the same buffer. Both PE and PEW281A were more susceptible to trypsin digestion in the presence of HSP (Figure 6). Although some degradation of nicked toxin was observed in the absence of HSP at higher trypsin concentration as was seen in Figure 4, more fragments were produced in the presence of HSP at all concentrations of trypsin tested. Taken together, these data indicate that the factor(s) necessary for both toxin unfolding and the reduction of the Cys265−287 disulfide bond are present in the HSP fraction of cells. The nature of these components is as yet unknown.

DISCUSSION

PE interacts with mammalian cells in a multistep process that begins with receptor-mediated endocytosis and ends with the ADP-ribosylation of cytoplasmic EF-2 leading to apo-

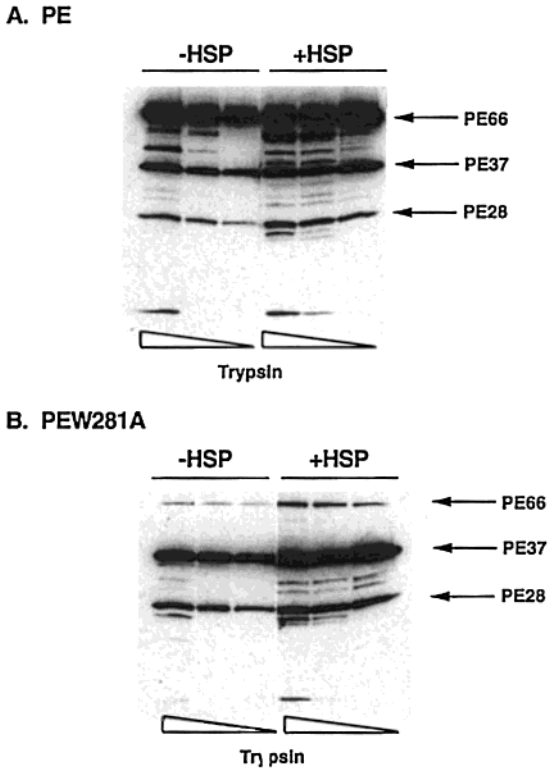

FIGURE 6: The HSP fraction can mediate toxin unfolding. (A) Furin-nicked PE was incubated with HSP from CHO cells in 100 mM HEPES, pH 7.5 at 37 °C. After incubation with HSP, the samples were treated with trypsin. The digested proteins were separated by reducing SDS−PAGE, immunoblotted with affinity-purified anti-PE, and visualized by chemiluminescence. (B) Nicked PEW281A treated as in panel A.

ptotic cell death. A key feature of this pathway is the reduction and physical separation of the C-terminal enzymatic fragment from the remainder of the toxin. Within cells this is accomplished by furin-mediated cleavage at Arg279 followed by the reduction of the disulfide bond linking cysteines 265 and 287. Here we have used furin-nicked PE to evaluate conditions that promote disulfide bond reduction leading to fragment separation. We report that reduction of PE requires an unfolding step that allows access to cellular reductants. Once unfolded, PDI can mediate the reduction of nicked PE. However, PDI by itself does not have the ability to unfold the toxin. We fractionated cells and sought cellular components that could mediate reduction of the Cys265−287 disulfide bond. Membranes from toxin-sensitive cells, but not the soluble fraction, mediated the combined unfolding and reduction steps. We used increased susceptibility to trypsin as an indicator of an unfolded state and production of PE37 as evidence of reduction.

A requirement for proteolytic processing followed by the reduction of a key intrachain disulfide bond is shared by many bacterial and some plant toxins. The heavy and light chains of botulinum and tetanus toxins are held together by a key disulfide bond. Reduction releases the enzymatically active light chain (*39*, *40*). The disulfide bond in the A chain of Shiga toxin appears to play an important role in stabilizing the enzymatic fragment after furin-mediated cleavage in the endosome (*41*). The intrachain disulfide bond holds the A1 and A2 chains together as the toxin moves from the endosome to the Golgi compartment. A mutant of Shiga toxin lacking this disulfide linkage was more susceptible to

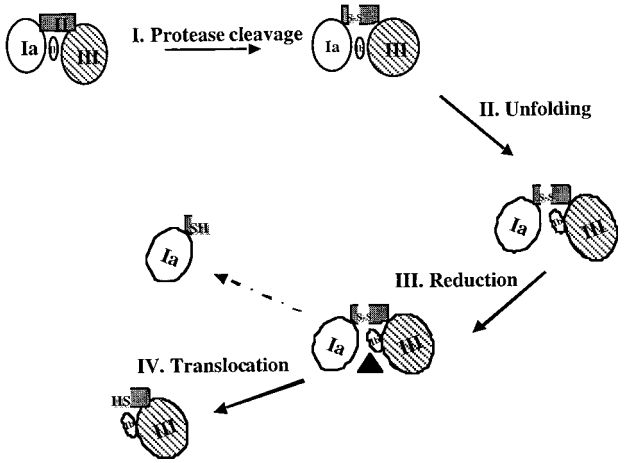

FIGURE 7: Postendocytic events in PE intoxication. An updated model of events in processing of PE after receptor-mediated endocytosis. The data presented here indicate that an unfolding event (II) mediated by a molecular chaperone must occur before the Cys265−287 disulfide bond is accessible to the reductant (III). The reductant (▲) may be PDI.

protease degradation and less cytotoxic to T47D cells (*41*). Recently, it was reported that the proteolytically nicked A subunit of cholera toxin (CTA) was reduced intracellularly by the redox enzyme PDI, most likely in the ER (*31*). In addition, Majoul et al. (*42*) observed that the disulfide bond linking the A1 and A2 fragments of CTA is reduced at glutathione concentrations that approximate the concentrations found in the ER. The conclusion from this study was that PDI augmented but was not required for the reduction of CTA. In the case of CTA, to be competent for reduction the toxin must be membrane-bound (*31*). Diphtheria toxin (DT) translocates directly from a low-pH endocytic compartment to the cytosol (*43*, *44*). However, like PE, there is a requirement for reductive release of the ADP-ribosylating fragment (DTA). In studying this, Ryser et al. (*45*) found that DT cytotoxicity was diminished by inhibitors of cell surface sulfhydryls. More recently, Papini et al. (*46*) have proposed that the reduction of the intrachain disulfide in DTA is the rate-limiting step in translocation to the cytosol. However, it remains unclear whether reduction occurs before or after insertion of DT into the cell membrane at the initiation of translocation. In addition to the native disulfide bond joining Cys186−201, Falnes et al. (*47*) engineered a "constraining" disulfide bond into DT. Despite cellular reduction of the native disulfide bond and release of DTA, the newly engineered bond remained refractory to reduction. This resulted in poor unfolding during the translocation step and loss of toxicity. While a unifying principle for the role of disulfide bonds in toxin biology has not been established, there may be common features. It appears that intrachain disulfide bonds stabilize key segments of the toxin until just prior to the translocation step. Reduction may be the trigger that allows unfolding to begin.

Structure−function studies and experiments studying PE's interactions with mammalian cells have produced models of its mode of action. Data on reduction now can be used to update this model (Figure 7). Receptor binding leads to endocytic uptake into an acidic endosomal compartment and cleavage by furin (Figure 7, step I, protease cleavage). Furin-nicked PE appears relatively stable (*9*). But under the mildly acidic condition of the endosome, we speculate that there is

exposure of core hydrophobic residues. Possibly, this leads to binding by chaperone proteins (Figure 7, step II, unfolding), which in cooperation with a reductase can produce reduced toxin fragments of 28 and 37 kDa (Figure 7, step III, reduction). The 37 kDa fragment has enzymatic activity and a functional KDEL-like sequence at its COOH-terminus. While the role of this KDEL-like sequence in promoting cellular reduction has not been fully clarified, preliminary data suggest that reduction is independent of KDEL. However, once reduction is complete, KDEL may be used to retrieve the reduced PE37 to the ER where it unfolds completely prior to translocation to the cell cytosol (Figure 7, step IV, translocation).

Most bacterial toxins that have a "translocation" function also have a key disulfide bond that must be reduced prior to reaching the cytosol. We speculate that the function of this disulfide bond is to retain core hydrophobic residues in a solvent-inaccessible state until unfolding is initiated. For PE and other toxins that interact with the ER, unfolding may be aided by chaperones. For DT and toxins that translocate directly from acidic endosomes, hydrophobic residues may move directly into the lipid bilayer.

Here we describe a novel step in PE biology, i.e., the transition from a stable nicked form to an unfolded (or partially unfolded) form. This step is necessary to allow access to and hydrolysis of a key disulfide bond. Because many cells are sensitive to PE-mediated killing, we speculate that the toxin unfolding activity will be a widely distributed protein.

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